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Reduced expression of glutamate transporter EAAT2 and impaired glutamate transport in human primary astrocytes exposed to HIV-1 or gp120

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Abstract

L-Glutamate is the major excitatory neurotransmitter in the brain. Astrocytes maintain low levels of synaptic glutamate by high-affinity uptake and defects in this function may lead to neuronal cell death by excitotoxicity. We tested the effects of HIV-1 and its envelope glycoprotein gp120 upon glutamate uptake and expression of glutamate transporters EAAT1 and EAAT2 in fetal human astrocytes in vitro. Astrocytes isolated from fetal tissues between 16 and 19 weeks of gestation expressed EAAT1 and EAAT2 RNA and proteins as detected by Northern blot analysis and immunoblotting, respectively, and the cells were capable of specific glutamate uptake. Exposure of astrocytes to HIV-1 or gp120 significantly impaired glutamate uptake by the cells, with maximum inhibition within 6 h, followed by gradual decline during 3 days of observation. HIV-1-infected cells showed a 59% reduction in V_{max} for glutamate transport, indicating a reduction in the number of active transporter sites on the cell surface. Impaired glutamate transport after HIV-1 infection or gp120 exposure correlated with a 40–70% decline in steady-state levels of EAAT2 RNA and protein. EAAT1 RNA and protein levels were less affected. Treatment of astrocytes with tumor necrosis factor-α (TNF-α) decreased the expression of both EAAT1 and EAAT2, but neither HIV-1 nor gp120 were found to induce TNF-α production by astrocytes. These findings demonstrate that HIV-1 and gp120 induce transcriptional downmodulation of the EAAT2 transporter gene in human astrocytes and coordinately attenuate glutamate transport by the cells. Reduction of the ability of HIV-1-infected astrocytes to take up glutamate may contribute to the development of neurological disease.

Keywords: HIV-1; gp120; Astrocytes; Glutamate uptake; TNF-α; Neuropathogenesis; HAD

Introduction

Dementia is a major complication of HIV-1 infection and an AIDS defining illness that until recently afflicted about a third of AIDS patients (Lipton and Gendelman, 1995; Navia et al., 1986; Navia and Price, 1987). The incidence of HIV-1-associated dementia (HAD) declined after the introduction of highly active antiretroviral therapies, but it still reaches 10% (Sacktor et al., 2001). The pathological hallmarks of HAD include neuronal loss, reactive gliosis, and white matter pallor (Budka, 1991; Everall et al., 1991; Navia et al., 1986; Sharer et al., 1986). HIV-1 infects infiltrating macrophages, microglial cells, and astrocytes in the brain (Koening et al., 1986; Ranki et al., 1995; Saito et al., 1994) but it is rarely found in neurons (Wiley et al., 1986), suggesting indirect viral action in neuronal cell loss. It has been suggested that cellular and viral products made by productively infected macrophages and microglial cells, in-

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cluding TNF-α, nitric oxide, gp120, and Tat, trigger inflammatory and neurotoxic reactions leading to HAD (Kaul et al., 2001; Lipton and Gendelman, 1995). Consistent with this hypothesis, HIV-1-infected human macrophages transplanted into mouse brain induced histopathological damage reminiscent of HAD (Persidsky et al., 1996). However, not all of the neuropathogenic effects of HIV-1 can be explained by productive HIV-1 infection in the brain. HAD is not always found in individuals with high virus burdens in the brain and is sometimes found in persons with low numbers of HIV-1-infected macrophages or microglial cells (Brew et al., 1995; Glass et al., 1995). Although X4 and not R5 virus is able to induce neuronal apoptosis in vitro (Ohagen et al., 1999; Zheng et al., 1999), many studies indicate that R5 HIV-1 strains predominate in the brain, consistent with their replication in monocytic cells (Korber et al., 1994; Power et al., 1994, 1998). Finally, some CNS isolates of HIV-1 poorly utilize CCR5 or preferentially replicate in T lymphocytes or in astrocytes (Canki et al., 1997; Smit et al., 2001). Together these findings indicate complexity in host cell tropism and receptor utilization by CNS-derived or neurotropic HIV-1 and in viral effects on its host cells in the brain.

A number of laboratories including ours have investigated astrocytes as targets for HIV-1 infection and pathology in the brain (reviewed by Brack-Werner, 1999; Conant et al., 1998). Although initially controversial (Gosztonyi et al., 1994; Wiley, 1996; Wiley et al., 1986), HIV-1 infection of astrocytes in vivo has been documented in many studies (An et al., 1999; Bagasra et al., 1996; Nuovo et al., 1994; Ranki et al., 1995; Saito et al., 1994; Stoler et al., 1986; Takahashi et al., 1996; Tornatore et al., 1994a). We have recently confirmed these findings by demonstration of HIV-1 DNA in individual astrocytes isolated from autopsy brain tissue by laser capture microdissection (Trillo-Pazos et al., 2003). Some reports, including ours, indicate that the frequency of HIV-1-positive astrocytes can reach about 1% in specific regions (Takahashi et al., 1996; Trillo-Pazos et al., 2003). The actual proportion of HIV-1-infected astrocytes varies by brain region analyzed and severity of CNS disease (Saito et al., 1994; Takahashi et al., 1996; Wiley et al., 1986) and it is higher in pediatric cases than in adults (Saito et al., 1994; Trillo-Pazos et al., 2003). The variability of HIV-1 detection in astrocytes in vivo likely contributes to the differences in opinion regarding the potential role of this infection in neuropathogenesis (Brack-Werner, 1999; Conant et al., 1998; Wiley, 1996).

In vivo, HIV-1-infected astrocytes express predominantly viral regulatory genes rather than structural genes associated with virus production (Ranki et al., 1995; Takahashi et al., 1996). This, and the high prevalence of gliosis in HAD (Budka, 1991), suggest that astrocytes survive HIV-1 infection in vivo and may carry virus for extended periods of time. Astrocytes engaged in apoptosis have also been described by some investigators (Shi et al., 1996; Thompson et al., 2001) but not by others (Hery et al., 1997; Krajewski et al., 1997). Consistent with observations in patients' tissues, HIV-1 infection of astrocytes in culture is low-productive, persistent, and noncytolytic (Cheng-Mayer et al., 1987; Dewhurst et al., 1987; Kunsch et al., 1989; Nath et al., 1995; Tornatore et al., 1991, 1994b) but the cells clearly have the capacity to produce infectious progeny virus (Canki et al., 2001; Cheng-Mayer et al., 1987; Hatch et al., 1994). The interaction of HIV-1 or gp120 with primary human astrocytes was shown to be independent of CD4, the viral coreceptors, and galactosyl ceramide (Cheng-Mayer et al., 1987; Ma et al., 1994; Sabri et al., 1999) and it may be mediated by an unknown high-affinity receptor for gp120 (Ma et al., 1994). HIV-1 replication in astrocytes can be increased by treatment with TNF-α and other cytokines (Canki et al., 2001; Shahabuddin et al., 1992a; Tornatore et al., 1991), indicating responses to physiological stimuli similar to those observed in other HIV-1 host cells (Rosenberg and Fauci, 1989). In summary, human astrocytes have emerged as an abundant and potentially important target cell for HIV-1 in the brain, but one in which the characteristics of HIV-1 infection are very different from the highly productive interaction typical for T-lymphocytes, macrophages, and microglial cells.

The present work was designed to address the cellular aspects of this atypical virus–cell interaction to determine the consequences of HIV-1 infection upon astrocyte functions. These essential functions include maintaining brain homeostasis, regulating the levels of extracellular glutamate, serving as a component of the blood-brain barrier, and responding to pathogens and brain injury (Danbolt, 2001; Dong and Benveniste, 2001; Verkhratsky et al., 1998). Recent research also suggests that astrocytes play a critical role in neuronal signal transmission by enhancing synaptic activity and strength, increasing the number of synapses, and modulating neuronal activity (Beattie et al., 2002; Iino et al., 2001; Oliet et al., 2001; Ullian et al., 2001). Disruption of any of these functions by HIV-1 could have significant impact on the progression of HIV-1 disease in the brain. We began to investigate the effect of HIV-1 and gp120 upon Na^+-coupled transport of L-glutamate and the expression of glutamate transporters mediating this uptake in primary human astrocytes. Five glutamate transporters (EAAT1–5) have so far been identified in humans; of these, EAAT1 and EAAT2 are expressed predominantly in astrocytes in the CNS and are believed to mediate most glutamate uptake in the brain (Anderson and Swanson, 2000; Danbolt, 2001; Gegelashvili and Schousboe, 1997; Palacin et al., 1998; Robinson, 1999). Here we show that exposure of human fetal astrocytes to HIV-1 or gp120 in culture induces transcriptional downmodulation of the glial glutamate transporter EAAT2 and reduction in expression of EAAT2 protein, resulting in extensive and lasting disruption of glutamate uptake by astrocytes.

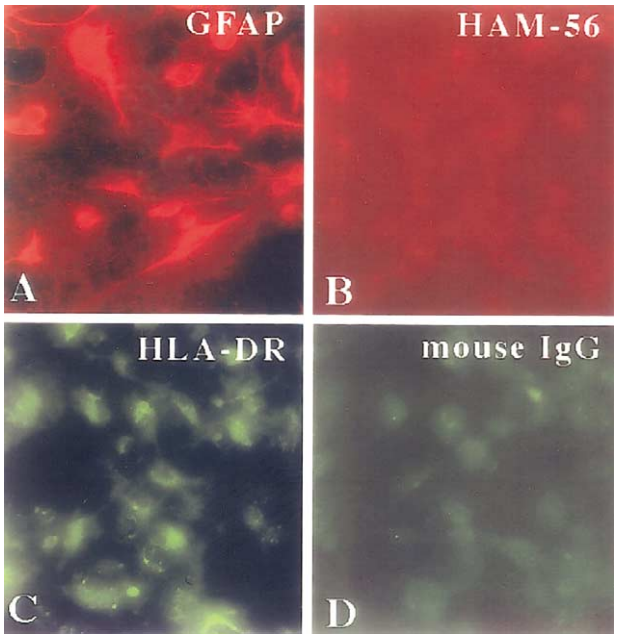

Fig. 1. Expression of selected cellular markers by human fetal astrocytes in culture. Astrocytes of 16 weeks gestational age were cultured on cover slips and stained for the designated proteins as described under Materials and methods. Control mouse immunoglobulin was used as the primary antibody in (D).

Results

Human fetal astrocytes express EAAT1 and EAAT2 transcripts and proteins and exhibit specific, high-affinity glutamate transport activity

Although HAD is a postnatal disease, fetal brain is the only practical source of primary human astrocytes in the numbers needed for experimental HIV-1 infection and biochemical and molecular studies. On that basis we adopted human fetal astrocytes as a model for this research. Astrocyte cultures were established from abortus brain tissue by a previously described procedure that permits isolation of an essentially homogeneous population of astrocytes (Bencheikh et al., 1999; Zheng et al., 1999). Fig. 1 shows photomicrographs from a typical culture used in the present studies after staining cells for expression of GFAP, the canonical astrocyte marker; HAM-56, a marker expressed by monocytic cells including microglial cells; and HLA-DR, which is expressed by both cell types after activation. Essentially all cells in culture expressed high levels of GFAP and HLA-DR, but no detectable HAM-56, indicating that they consisted mostly of astrocytes. There was no detectable nonspecific staining (Fig. 1D). Batches of astrocytes were routinely monitored for expression of these antigens with similar results.

Next we confirmed that fetal astrocytes express the two known glial glutamate transporters EAAT1 (GLAST) and EAAT2 (GLT-1) and tested whether the cells are capable of specific glutamate transport. Fig. 2 shows the results of EAAT protein and RNA analyses in astrocytes. All cell extracts were standardized for expression of a housekeeping protein, α-tubulin, or a housekeeping RNA, ribosomal small subunit protein 9 (RPS), prior to evaluation of transporter expression (Fig. 2). Since expression of glutamate transporters is developmentally regulated (Bar-Peled et al., 1997; Shibata, 1997), initial experiments determined the presence of EAAT2 in cells of different gestational age (Fig. 2A). We found that astrocytes expressed the EAAT2 protein of the expected approximate mass of 73–75 kDa and that EAAT2 expression increased between 16 and 19 weeks of gestation (Fig. 2A), consistent with the observed changes in transporter expression during development (Bar-Peled et al., 1997). Further studies described here employed cells of 16 weeks or greater gestational age. We also compared two different batches of astrocytes (18 weeks gestation time) for expression of EAAT1 and EAAT2 and found that EAAT2 was more readily detectable than EAAT1 (Fig. 2B). The difference is probably due to different sensitivities of anti-EAAT1 and EAAT2 antibodies, as EAAT1 is known to be expressed well by prenatal astrocytes (Bar-Peled et al., 1997). To confirm these studies at the RNA level, we performed Northern blot analysis using RNA extracted from three further astrocyte populations (Fig. 2C). Both the 4-kb EAAT1 and the 12-kb EAAT2 RNA (Arriza et al., 1994) were detectable in cells from three different donors. The glioma cell line U87 did not produce detectable EAAT2 RNA but, consistent with previous reports (Ye et al., 1999), expressed low levels of EAAT1 RNA.

To determine whether astrocytes are capable of specific glutamate transport, we employed the assay of L-[^{3}H]-glutamate uptake, essentially as previously described (Fine et al., 1996). We first determined the time course and specificity of L-[^{3}H]-glutamate uptake. As shown in Fig. 3, astrocytes internalized glutamate linearly with time up to 15 min after exposure. Further assays were performed within this linear phase of uptake. The specificity of the assay was demonstrated by inhibition of glutamate uptake in the presence of 250-fold excess of unlabeled EAAT1/EAAT2 ligands L-glutamate or L-aspartate, or after preincubation with a conformationally constrained substrate analog L-*trans*-2,4-PDC (inset in Fig. 3). Similar results were obtained in five experiments with different astrocyte preparations. Analysis of glutamate uptake kinetics in fetal astrocytes, similar to that shown later in this work (Fig. 6), determined the V_{max} for transport of 4–5 nmol glutamate/mg protein/min and K_m of 19–20 μM, both within the range reported for Na^+-dependent, high-affinity glutamate transport in primary human and rodent astrocytes or synaptosomal vesicles (Danbolt, 2001; Fine et al., 1996; Robinson, 1998). Together, these results indicate that fetal human astrocytes express glutamate transporters EAAT1 and EAAT2 and can be reliably monitored for specific glutamate uptake and its inhibition in culture.

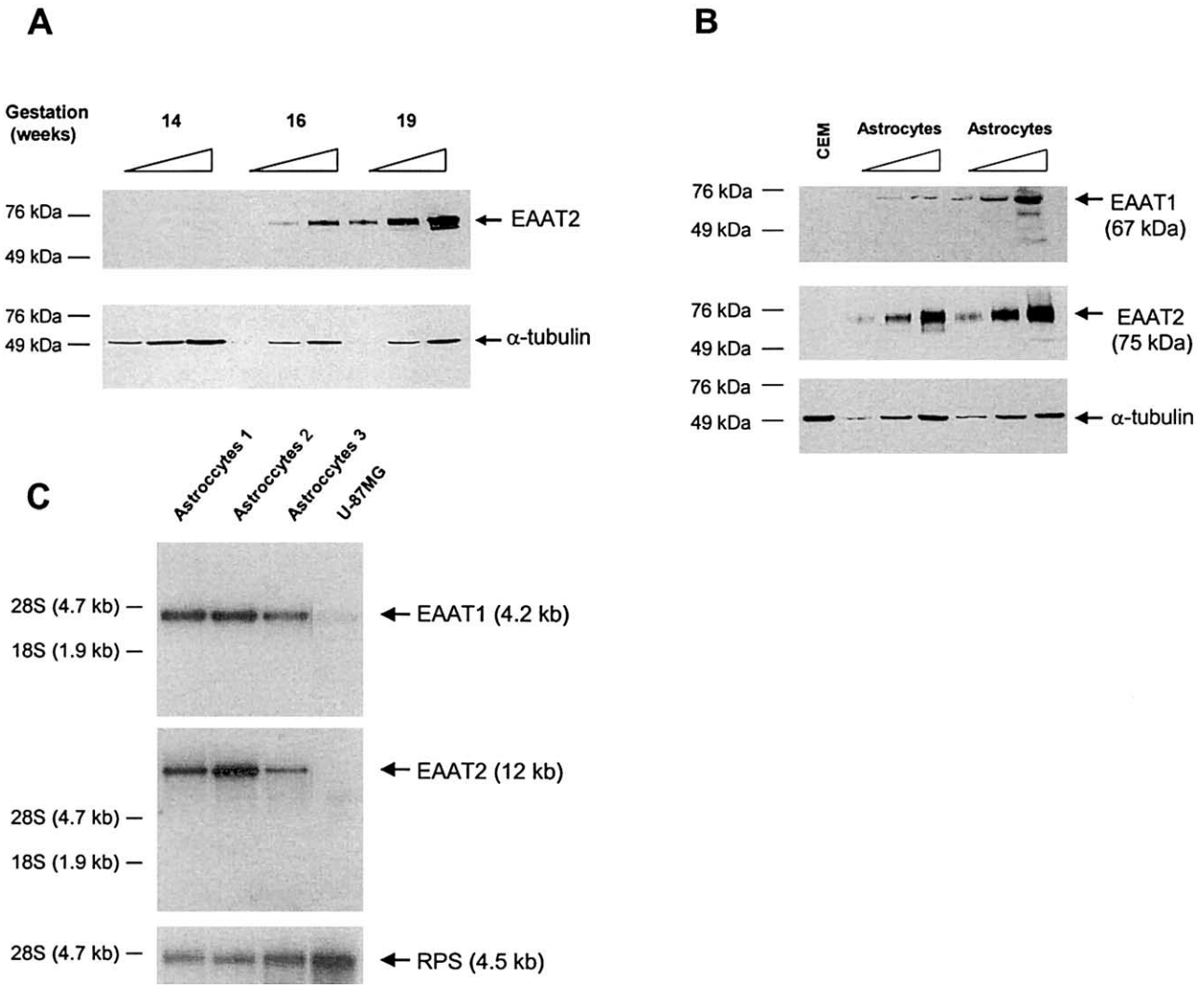

Fig. 2. Expression of glutamate transporters EAAT1 and EAAT2 by human fetal astrocytes in culture. (A) 5, 10, and 20 μg protein per lane from three populations of astrocytes at the indicated gestational ages were subjected to immunoblotting using anti-EAAT2 or anti-α-tubulin as described under Materials and methods. (B) 5, 10, and 20 μg protein per lane from two different populations of astrocytes of 18 weeks gestational age or 10 μg protein from negative-control CEM T lymphoblastoid cells were subjected to immunoblotting using anti-EAAT1, anti-EAAT2, or anti-α-tubulin as described under Materials and methods. (C) One microgram of total RNA isolated from three different populations of astrocytes of 16–18 weeks gestational age or from U87MG glioma cells was subjected to Northern blot analysis probing for EAAT1, EAAT2, and RPS transcripts as described under Materials and methods.

HIV-1 infection or exposure to gp120 attenuates glutamate uptake by primary human astrocytes

Next we investigated the effects of HIV-1 and recombinant gp120 protein on glutamate uptake by astrocytes and on EAAT1 and EAAT2 expression. Astrocytes were exposed to HIV-1/NL4-3 at 1 infectious units (IU) per cell and virus infection was monitored by measuring the levels of extracellular and intracellular HIV-1 p24 capsid antigen over 10 days in culture (Fig. 4). As previously reported by others as well as ourselves (Bencheikh et al., 1999; Canki et al., 2001; Nath et al., 1995; Tornatore et al., 1991), and analogous to infection observed in astrocytes in the brain (Saito et al., 1994; Takahashi et al., 1996), infection of astrocytes by HIV-1 was low-productive and noncytopathic, peaking at about 50,000 pg p24 per 10^6 cells (Fig. 4). To determine functional consequences of exposure, astrocytes were tested for their ability to take up L-[H^3]-glutamate at various times after HIV-1 infection or treatment with glycosylated gp120 at 1 nM. Fig. 5A and B shows typical experiments revealing that within 6 h both HIV-1 infection and $gp120_{MN}$ exposure significantly inhibited glutamate uptake by astrocytes. At this time, the inhibition was about 70% in HIV-1-infected cells and nearly 60% in gp120-treated cells. Inhibition declined over the course of observation, although it was still measurable 3 days after treatment at 19 ± 7% for HIV-1 and 25 ± 8% for gp120 (Fig. 5A and B). We tested the specificity of the response and found that X4 HIV-1/NDK and R5 HIV-1/ADA infection also inhibited glutamate uptake by astrocytes at levels similar to infection by NL4-3, but that exposure to serum albumin or ovalbumin at 100-fold excess over gp120 had no

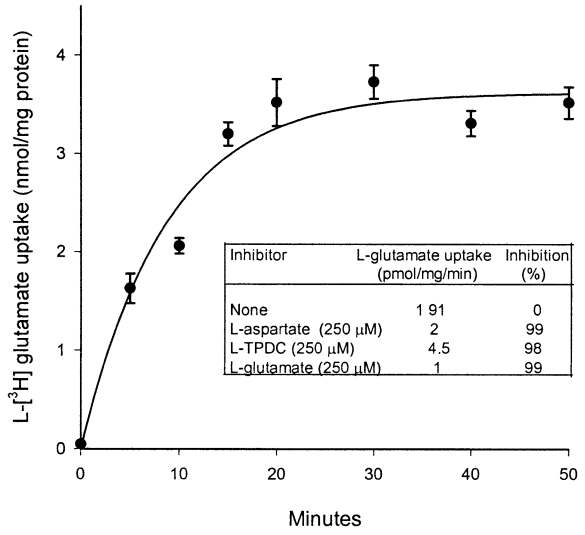

Fig. 3. Time course of L-[^{3}H]-glutamate uptake in fetal astrocytes. Cell-associated radiolabeled glutamate was measured at the indicated times as described under Materials and methods. The activity of glutamate transport inhibitors (inset) was assayed after 10 min of exposure and uptake.

effect upon glutamate uptake at any time (not shown). The inhibition of glutamate uptake in astrocytes was virus and gp120 dose-dependent, reaching maximum levels with 1 IU of HIV-1/NL4-3 per cell and 1 nM $gp120_{MN}$. To determine the reproducibility of the observed responses, we tested a large number of astrocyte pools for glutamate uptake after exposure to NL4-3 or $gp120_{MN}$ (Fig. 5C–D). Overall, at the doses used, infection by NL4-3 was more inhibitory than was exposure to gp120 at their optimal doses. At the 6 h time point, the inhibition by HIV-1 was 75.8 ± 13.8% (mean ± SD, $n = 20$) and gp120-mediated effect was 58.0 ± 17.9% ($n = 10$) ($P = 0.001$, paired Student's t test). The respective values at 24 h after treatment were 54.3 ± 15.9% ($n = 15$) and 36.5 ± 15.4% ($n = 10$) with $P = 0.05$. Together, the data shown in Fig. 5 using cells from more than 25 different brains indicate that HIV-1 and its envelope glycoprotein have a pronounced, specific, long-lasting, and reproducible inhibitory effect upon the ability of human astrocytes to take up extracellular glutamate.

HIV-1-induced impairment of glutamate uptake correlates with reduction in glutamate transport velocity (V_{max}) and downmodulation of the expression of EAAT2 protein and RNA in primary human astrocytes

A variety of physiological and pathogenic stimuli were shown to regulate glutamate transport at multiple levels, including control of transcription, mRNA processing, and translation of EAAT genes, posttranslational modifications of EAAT proteins, or changes in the ionic or electrophysiological environment of astrocytes (Danbolt, 2001; Gegelashvili et al., 1997; Maragakis and Rothstein, 2001). To begin to investigate the mechanism of the observed HIV-1 and gp120 effects, we first characterized the biochemical properties of glutamate transport activity to determine whether HIV-1 exposure altered transport constants V_{max} and K_m (Fig. 6). Concentration-dependence of L-[^{3}H]-glutamate uptake was tested in HIV-1 infected and control astrocytes; the results are shown in the insets in Fig. 6A and B. Eadie–Hofstee transformation of these data was used to derive transport constants (Fig. 6). HIV-1 exposure decreased the V_{max} value from 4.6 ± 0.4 in control cells to 1.9 ± 0.3 nmol/mg protein/min, a 59% decline, but had no significant effect on the K_m (19.1 ± 2.3 μM in control and 23.75 ± 2.0 μM in HIV-1-treated cells), indicating that HIV-1 infection reduced the number of glutamate transporters functional at the astrocyte surface without altering their affinity for glutamate.

To determine whether the observed inhibition of glutamate transport correlates with expression of transporter protein or RNA, we tested the steady-state expression of EAAT1 and EAAT2 proteins by Western blot and levels of transporters mRNA by Northern blot (Fig. 7). Astrocytes were treated with HIV-1/NL4-3, and for comparison, with a known inducer of EAAT2 expression 8-bromo-cyclic adenosine monophosphate (8-Br) (Schlag et al., 1998) and an inhibitor of glutamate uptake, TNF-α (Fine et al., 1996); the cells were tested for EAAT1 and EAAT2 protein and RNA expression 24 h after treatment. Protein and RNA inputs were standardized by the levels of α-tubulin and RPS, respectively, and relative intensities of the bands were evaluated by densitometry. As shown in Fig. 7A, EAAT2 protein levels were clearly reduced after HIV-1 infection relative to untreated cells, but there was only a small reduction in EAAT1 protein. In contrast, TNF-α strongly inhibited the expression of both proteins. As expected, 8-Br induced both EAAT1 and EAAT2, although the two-fold induction of EAAT2 was smaller than reported in studies using rat astrocytes (Schlag et al., 1998). The observed changes in steady-state levels of EAAT1 and EAAT2 mRNA in HIV-1, 8-Br, or TNF-α-treated astrocytes (Fig. 7B) closely paral-

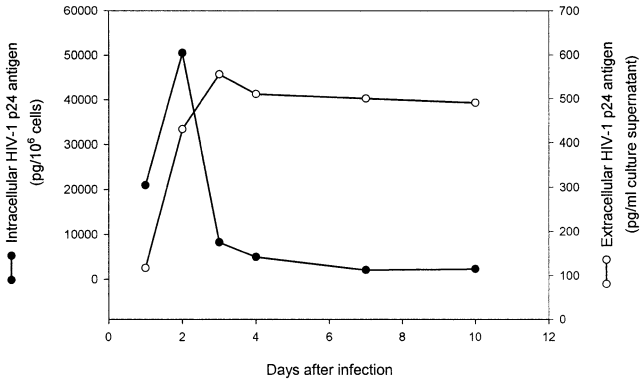

Fig. 4. HIV-1 infection of astrocytes monitored by p24 capsid antigen production. Astrocytes were infected with cell-free NL4-3 at 1 IU per cell and intracellular and extracellular viral capsid antigen p24 levels were monitored over time after infection.

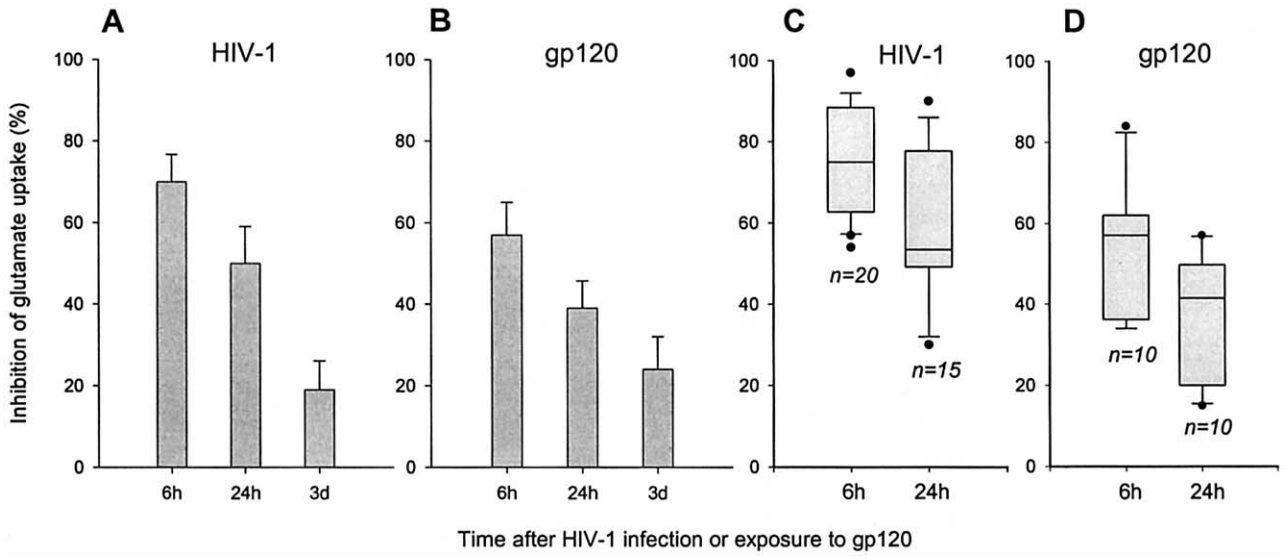

Fig. 5. Inhibition of L-[^{3}H]-glutamate uptake in fetal astrocytes by HIV-1 and gp120. Astrocytes were exposed to HIV-1 (A,C) or gp120 (B,D), and glutamate uptake was evaluated as described under Materials and methods. The results shown are the means ± SEM of duplicate or triplicate wells of % inhibition relative to cells treated with medium in parallel. L-[^{3}H]-glutamate assays were conducted for 10 min. (A) (B) The time course of the effects of virus and gp120 upon glutamate uptake was evaluated by testing astrocytes of 16 weeks gestational age at the indicated times after treatment with HIV-1 at 1 IU per cell (A) or gp120 at 1 nM (B). (C) (D) The effects of HIV-1 (C) or gp120 (D) upon glutamate uptake were tested in multiple independent astrocyte populations 6 and 24 h after treatment as indicated. The data were pooled and plotted using the SigmaPlot statistical package for box plots. The line inside the box denotes 50%. The box extents denotes 75%; the capped bars denote 90%, and the dots flanking the box denote 95% of data values.

leled those seen at the protein level. Twenty-four hours after HIV-1 infection there was a pronounced decline in the primary transcript of EAAT2 relative to control RPS transcripts. There was no visible effect of HIV-1 on EAAT1 RNA expression, consistent with its minimal effects upon EAAT1 protein levels (Fig. 7A). Similar to induction of

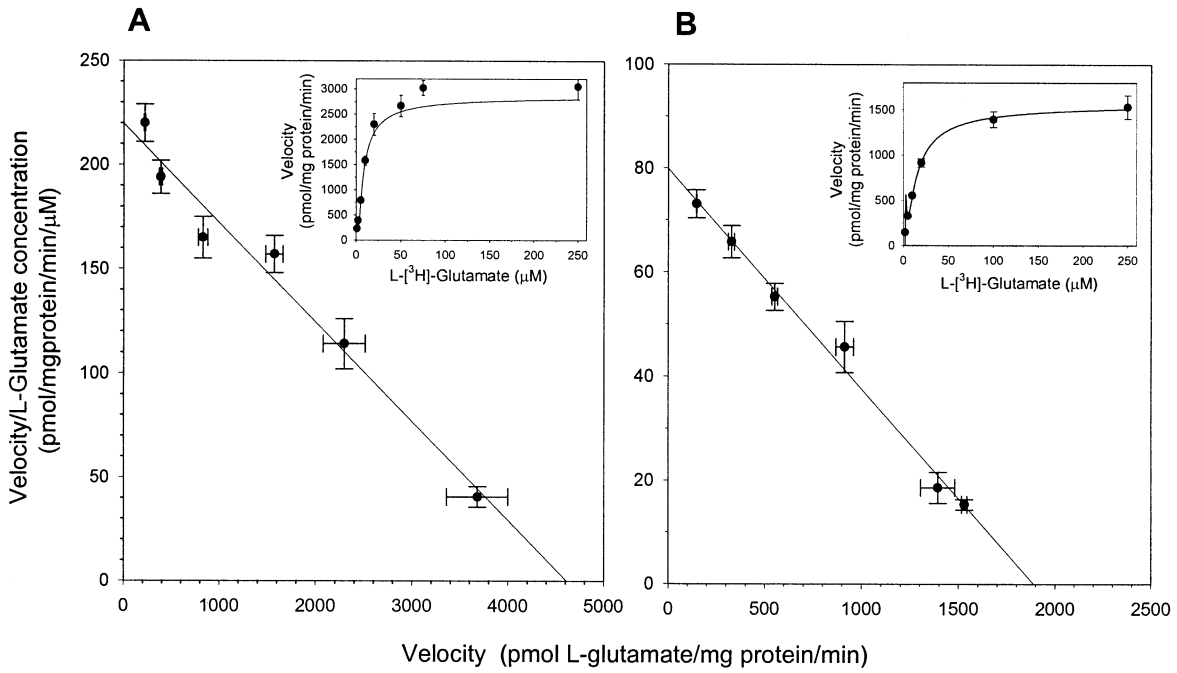

Fig. 6. HIV-1 reduces the V_{max} for glutamate transport in fetal astrocytes. Fetal astrocytes were treated with medium (A) or infected by HIV-1 (B) and 24 h later glutamate uptake assays were performed in the presence of increasing concentrations of unlabeled L-glutamate as described under Materials and methods. Results are shown as the Eadie–Hofstee transformations of the concentration dependence of the high-affinity L-[^{3}H]-glutamate transport in HIV-1-infected fetal astrocytes and they represent the means ± SEM of three independent experiments performed in triplicate. The K_m and V_{max} values are listed in the text. Insets: Saturation kinetics of the same data in Michaelis–Menten plots.

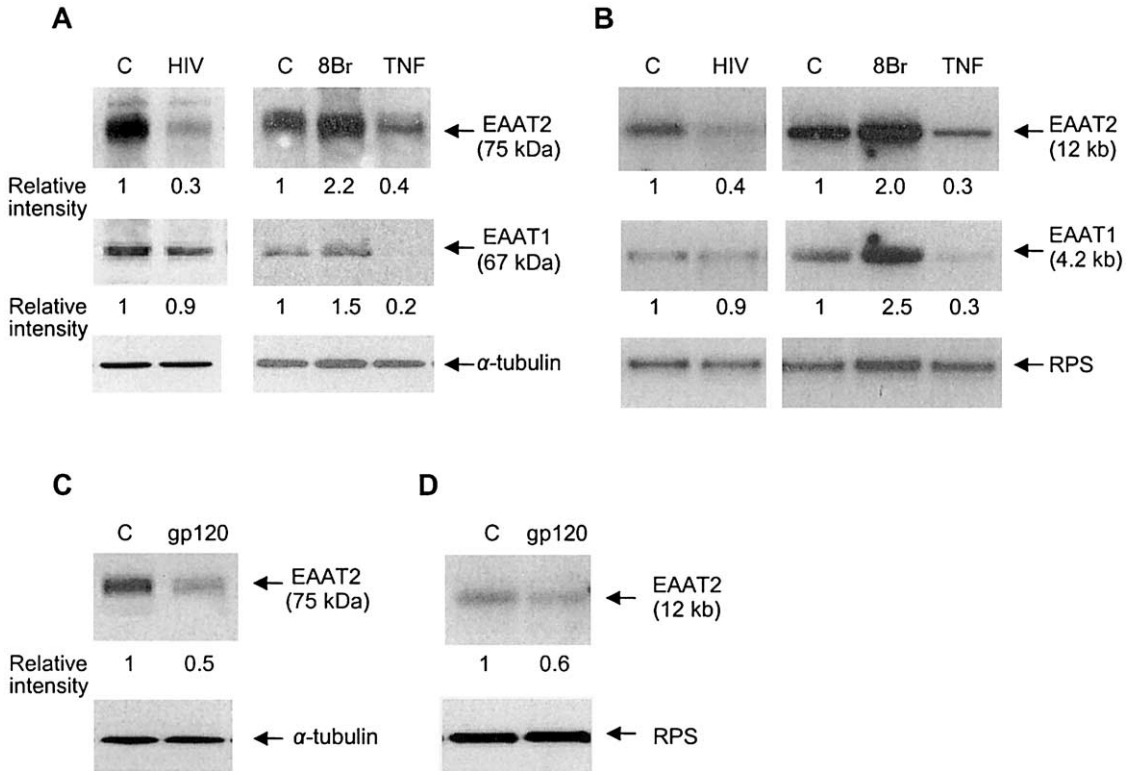

Fig. 7. Inhibition of EAAT protein and RNA expression in fetal astrocytes after HIV-1 infection, exposure to gp120, or treatment with TNF-α or 8-Br-c-cAMP. Fetal astrocytes of 18 weeks gestational age were infected with NL4-3 or treated with gp120, TNF-α, or 8-Br. Six hours after treatment, cells were harvested for protein and RNA extraction and detection by Western (A and C) or Northern (B and D) blot, respectively, as described under Materials and methods. The intensities of protein and RNA bands were determined by densitometry as described; relative intensities are listed under the respective bands.

transporter proteins, 8-Br induced both EAAT1 and EAAT2 RNA and TNF-α inhibited expression of EAAT1 and EAAT2 (Fig. 7B). Similar to HIV-1-infected cells, astrocytes exposed to $gp120_{MN}$ for 24 h also showed significant reduction in steady-state levels of EAAT2 protein and RNA (Fig. 7C and D, respectively). No significant effect on EAAT1 protein or RNA was seen in gp120-treated cells (not shown). Comparable results were obtained in three independent experiments using different pools of astrocytes. Overall, our results indicate that HIV-1 and gp120 inhibit EAAT2 gene transcription, resulting in reduced EAAT2 RNA and protein expression, reduced surface glutamate transporters, and impaired uptake of glutamate. We also show that TNF-α inhibits the expression of both EAAT1 and EAAT2 at the RNA and protein levels, consistent with its previously described effects upon glutamate uptake (Fine et al., 1996).

HIV-1 infection or exposure to gp120 does not induce secretion of TNF-α by astrocytes

TNF-α was shown to inhibit glutamate uptake in human astrocytes (Fine et al., 1996) and as we show here, TNF-α inhibited the expression of both EAAT1 and EAAT2 protein and RNA in astrocytes (Fig. 7). Because exposure to HIV-1 can induce TNF-α synthesis by macrophages and lymphocytes (Choe et al., 2001; Poli et al., 1990), we investigated HIV-1 and gp120 as potential inducers of TNF-α. Fetal astrocytes were either infected with HIV-1 or treated with gp120, or exposed to PMA or IL-1β, known inducers of NF-κB and HIV-1 replication in astrocytes (Shahabuddin et al., 1992b; Tornatore et al., 1991). In parallel, MDM serving as control were treated with PMA, IL-1β, or LPS, an activator of cytokine and chemokine production in MDM (Choe et al., 2001), or exposed to astrocyte supernatants, known to activate macrophages (Nottet et. al., 1995). All the systems were tested for the presence of TNF-α in culture supernatants 24 h after treatments (Fig. 8). The results clearly show that neither HIV-1 infection, nor gp120 exposure, nor any other treatments used induced secretion of TNF-α by human astrocytes. LPS and PMA, but neither IL-1β nor astrocyte supernatant, induced TNF-α synthesis by MDM.

Discussion

Glutamate is the major neurotransmitter in the brain and its removal from synapses by glutamate transporters is essential for signal transmission and prevention of excitotoxic

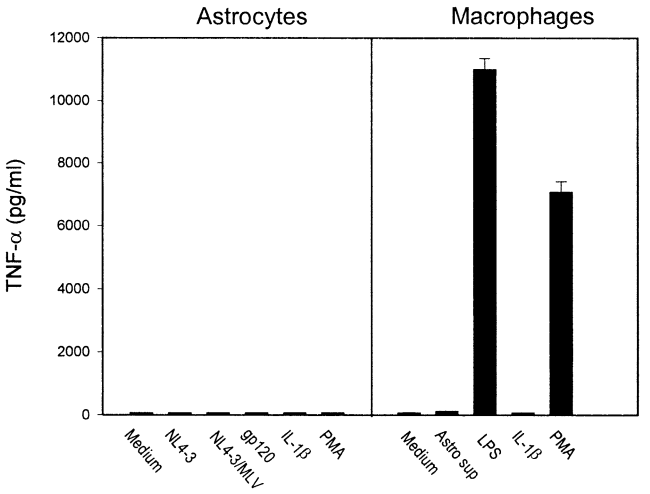

Fig. 8. TNF-α is not induced by HIV-1 or gp120 treatment of fetal astrocytes. Fetal astrocytes of 16 weeks gestational age or control MDM were treated with the indicated agents and extracellular TNF-α was assayed by ELISA.

death of neurons (Anderson and Swanson, 2000; Choi, 1988; Danbolt, 2001; Gegelashvili and Schousboe, 1997). Glutamate excitotoxicity has been proposed as part of the etiology in several neurological diseases including amyotropic lateral sclerosis, epilepsy, Alzheimer's disease, as well as HAD (Choi, 1988; Hansson et al., 2000; Kaul et al., 2001; Maragakis and Rothstein, 2001). In the present work, we demonstrate that exposure to HIV-1 or gp120 in culture induces rapid, extensive, and lasting reduction of glutamate transport by primary human astrocytes. Our data also indicate that the proximal cause of this defect is transcriptional downmodulation of the glial glutamate transporter EAAT2 and consequently EAAT2 protein. If astrocytes respond to native HIV-1 or gp120 in vivo as we observe in vitro, this virus-induced dysfunction may contribute to glutamate excitotoxicity and neurodegeneration associated with HAD.

Several functional and molecular attributes of HIV-1 and gp120 were investigated for their impact upon glutamate uptake inhibition. We found that the inhibition of glutamate uptake was extensive, reaching a peak of 70–90% at 6 h (Fig. 5A). At 6 h, HIV-1-mediated glutamate uptake inhibition averaged 76% and the gp120-mediated effect was 58%; 24 h after treatment inhibition was 54 and 36%, respectively, at the optimal doses for each agent. Statistical analysis of uptake results from up to 20 independent experiments with tissues from different donors and with different virus preparations (Fig. 5D) confirmed the generality and extent of the virus and gp120 effects. Although uptake results varied among individual experiments as indicated by the data spreads shown in Fig. 5D, the observed inhibitory trend was remarkably consistent across multiple experiments, especially considering the expected differences in genetic backgrounds of tissue donors and different gestation times of the fetal tissue used. Glutamate uptake inhibition was also observed during infection of feline astrocytes with feline immunodeficiency virus (FIV) (Billaud et al., 2002; Yu et al., 1998) and in human astrocytes cocultivated with HTLV-I-infected T cells (Szymocha et al., 2000). FIV causes an HAD-like disease in cats (Henriksen et al., 1995; Pedersen and Barlough, 1991) that was shown to be accompanied by increased glutamate levels and neuronal loss in the brain (Power et al., 1997) and HTLV-I is the etiologic agent of tropical spastic paraparesis/HTLV-1-associated myelopathy (Gessain et al., 1985; Osame et al., 1986), suggesting that disruption of astrocyte glutamate transport may be a common cytopathogenic activity of some neurotropic retroviruses. In addition, Benos and colleagues showed that treatment of rat astrocytes with exogenous gp120 altered ion fluxes across the plasma membrane and increased D-aspartate efflux (Benos et al., 1994a, b), while Holden and colleagues demonstrated that gp120 can activate the Na^+/H^+ exchangers, glutamate release, and $[Ca^{2+}]$ influx in human astrocytes (Holden et al., 1999), indicating that exposure to gp120 alone can affect excitatory amino acid transport in these cells.

The extent of HIV-1-mediated inhibition of glutamate uptake in astrocytes, with a mean peak of 76%, indicated that HIV-1 affected the great majority of cells in culture. This was surprising considering that HIV-1 infection of astrocytes is inefficient and generally fewer than 1% of the cells have detectable HIV-1 antigens or DNA in experimental (Bagasra et al., 1992; Bencheikh et al., 1999; He et al., 1997) or natural infection (Saito et al., 1994; Takahashi et al., 1996; Tornatore et al., 1994a; Trillo-Pazos et al., 2003). However, the finding that gp120 was comparable to infectious HIV-1 in its inhibition of glutamate transport (Fig. 5) indicates that the bulk of the inhibitory effects of HIV-1 can be attributed to the virus interaction with cell surface, prior to and independent of subsequent viral replication in the minority of cells. Indeed, inactivation of HIV-1 replication by irradiation did not affect its ability to inhibit glutamate uptake by astrocytes (not shown). The time course of inhibition also indicates its independence from virus replication: glutamate uptake was inhibited as rapidly as 1 h after HIV-1 exposure (not shown) and reproducible and maximal inhibition was observed at 6 h (Fig. 5A). The rapid kinetics of HIV-1 and gp120 effects in human cells contrast with the FIV system, in which inhibition of glutamate transport was apparent only a week after infection and was more pronounced with highly productive FIV/34TF10 than with the low-productive FIV/PPR, indicating dependence on FIV replication (Billaud et al., 2002; Yu et al., 1998). The difference between the two systems may reflect the fact that feline astrocytes are more permissive to FIV than are human astrocytes permissive to HIV-1 (Fig. 4) (Billaud et al., 2002; Tornatore et al., 1991, 1994a).

Analysis of changes in glutamate transport kinetics and expression of transporters induced in response to HIV-1 or gp120 (Figs. 6–8) permits us to draw some preliminary conclusions concerning the mechanisms of the effect. Our results suggest that the proximal cause of this inhibition is a

reduction in the number of functional glutamate transport sites on HIV-1-infected cells, as indicated by a 59% decline in V_{max} without change in K_m (affinity) for glutamate (Fig. 6). The reduction in V_{max} of glutamate transport correlated directly with the decline in the steady-state levels of EAAT2 mRNA and protein, with EAAT1 expression less affected (Fig. 7), indicating that HIV-1 and gp120 may act through downmodulation of EAAT2. However, the glutamate uptake assay employed here does not distinguish between EAAT1 and EAAT2, and so we cannot yet conclude that the observed marked decline in EAAT2 protein and RNA accounts fully for the decline in glutamate transport function. Studies are under way to distinguish between the activities of the two transporters in glutamate uptake assays by means of transporter-specific inhibitors and to determine the degradation rates of EAAT1 and EAAT2 proteins and RNA.

The cellular pathways responsible for HIV-1-mediated downmodulation of EAAT2 expression and glutamate transport inhibition remain to be investigated. We tested TNF-α as one attractive candidate to transduce HIV-1 response in astrocytes because HIV-1 can induce TNF-α synthesis in macrophages (Choe et al., 2001); exogenous TNF-α was shown to inhibit glutamate uptake through a reduction in V_{max} for glutamate transport (Fine et al., 1996), and activation of endogenous TNF-α by ligation of CXCR4 on astrocytes was reported to induce glutamate release (Bezzi et al., 2001). However, no TNF-α production was observed in HIV-1 or gp120-treated astrocytes under conditions where EAAT2 function and transcription were inhibited (Fig. 8). Moreover, the responses of astrocytes to TNF-α were markedly different from their responses to HIV-1. TNF-α required 24 h to achieve maximal inhibition of glutamate uptake (Fine et al., 1996) and we report here that it affected both EAAT1 and EAAT2 expression. HIV-1, in contrast, achieved maximal inhibition 6 h after infection and its effects were mediated mostly by changes in EAAT2 expression. Together, these experiments rule out TNF-α as the agent of inhibition of glutamate uptake induced by HIV-1 and gp120 in this system.

Our results presented here and elsewhere (Su et al., 2002, 2003) indicate that HIV-1 and gp120 exposure alter the transcriptional program of human astrocytes, resulting in profound functional changes exemplified here by impaired glutamate transport. Astrocytic maintenance of extracellular glutamate within narrow limits is critical for glutamergic signal transmission on the one hand and for preventing overexcitation of glutamate receptors and neuronal cell death on the other hand (Choi, 1988; Danbolt, 2001; Maragakis and Rothstein, 2001). Glutamate transport is tightly regulated through transporter expression by astrocytes during development, in the mature brain, and may be dysregulated during disease (Choi, 1988; Danbolt, 2001; Maragakis and Rothstein, 2001). Pathways of neuropathogenesis involving factors ranging from TNF-α and arachidonic acid to amyloid precursor protein appear to functionally converge in their disruption of glutamate transport in astrocytes, thereby reducing glutamate clearance and, potentially, causing neurodegeneration (Dreyer and Lipton, 1995; Fine et al., 1996; Li et al., 1997). Our data place HIV-1 and gp120 in this category of the potentially pathogenic downmodulators of glutamate transport in human astrocytes, suggesting that the interaction of astrocytes with HIV-1 or gp120 may directly contribute to glutamate excitotoxicity and neurodegeneration associated with HAD.

Materials and methods

Cells

Astrocytes were isolated from second trimester (14–19 weeks of gestational age) human fetal brains obtained from elective abortions in full compliance with NIH guidelines, as previously described (Bencheikh et al., 1999; Canki et al., 2001). Homogenous preparations of astrocytes were obtained using high-density culture conditions in the absence of growth factors in F12 Dulbecco's modified Eagles Medium (DMEM-F12) (GIBCO-Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS), penicillin, streptomycin, and gentamycin. Cells were maintained in this medium at $2–5 \times 10^4$ cells/cm^2 and subcultured weekly up to six times. Cultures were regularly monitored by immunofluorescence staining for expression of the astrocytic marker glial fibrillary acidic protein (GFAP) and HAM-56 to identify cells of the monocyte lineage. For these analyses, cells were cultured on coverslips and stained and evaluated as described (Canki et al., 2001), using primary antibodies rabbit anti-GFAP and anti-HAM-56 (both from Dako Co., Carpinteria, CA), and anti-HLA DR (Roche Molecular Biochemicals, Indianapolis, IN) and appropriate fluorochrome-labeled secondary antibodies (Sigma, St. Louis, MO). Only cultures that contained $\geq$99% GFAP-positive astrocytes and no detectable HAM-56-positive cells were used in these experiments (Fig. 1) and in Canki et al. (2001). Human monocytes were isolated from peripheral blood mononuclear cells and differentiated to macrophages (MDM) for 7 days prior to use as described (Choe et al., 2001). Other cells used in this study were the human embryonal kidney epithelial cell line 293T, used for HIV-1 propagation, and MAGI cells, a derivative of HeLa carrying the β-gal gene under the control of HIV-1 LTR and expressing HIV-1 receptors (Kimpton and Emerman, 1992), used as indicator cells for HIV-1 titration. Both cell lines were cultured in 90% DMEM/10% FCS supplemented with antibiotics and, for MAGI cells, 0.2 mg/ml G-418.

Viruses

The HIV-1 species primarily used in this work was NL4-3, an X4 laboratory clone that expresses all known HIV-1 proteins (Adachi et al., 1986). In some experiments, a molecular clone of the X4 isolate NDK from Zaire (Spire

et al., 1989) and a North American R5-isolate ADA (Gendelman et al., 1988) were used for comparison. Generally, NL4-3 virus stocks were prepared by culture of infected CEM cells; virus stocks were concentrated by high-speed centrifugation (12,000 *g*, 2 h, 4°C), resuspended in PBS and frozen at −80°C. Alternatively, NL4-3 and NDK were propagated by transfection of 15 μg of viral DNA into 1.5 $\times$ 10^6 293T cells as previously described (Bencheikh et al., 1999). Culture supernatants were harvested 72 h after transfection, filtered through a 0.45-μm Millipore filter, and stored at −80°C until use. ADA was propagated in MDM as described (Choe et al., 2001). Cell-free viral stocks were tested for HIV-1 p24 core antigen content by ELISA using HIV-1 Ag kit according to the manufacturer's instructions (Coulter, Hialeah, FL), for titers of infectious virus by MAGI assay (Kimpton and Emerman, 1992), and for the presence of TNF-α by Quantikine ELISA kit as described below. Culture supernatants contained 1–2 μg/ml of viral p24 protein and 1–2 $\times$ 10^6 IU per milliliter but no detectable TNF-α. NL4-3 virus preparations yielded similar results in astrocytes whether they were propagated in infected CEM cells or transfected 293T cells.

HIV-1 infection or exposure to gp120

Early passage astrocytes were cultured for 5–7 days until 75% confluence; the cells were washed in warm PBS and infected with HIV-1 at 1 IU per cell. Cell–virus mixtures were incubated for 6 h; the cells were then washed three times to remove input virus, cultured in fresh medium, and harvested at time points indicated in the figures. Control astrocytes were treated as described above but without HIV-1. Cells were similarly treated with full-length, recombinant, glycosylated $gp120_{MN}$ protein (NIH AIDS Research and Reagents Program, catalog no. 3927; produced by ImmunoDiagnostic, Inc.) at a final concentration of 1 nM or with control proteins at the concentrations indicated. Progress of viral infection was monitored by measurement of extracellular p24 in culture supernatant or intracellular p24 by ELISA.

Glutamate uptake assays

Astrocytes were plated in triplicate at 1 $\times$ 10^5 cells per well in 12-well tissue culture plates, grown for 6–8 days or 75% confluence, and equilibrated at 25°C in incubation medium: 0.125 M NaCl, 4.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 5 mM glucose, buffered to pH 7.4 with sodium phosphate. After addition of L-[^{3}H]-glutamic acid (DuPont NEN, Boston, MA; specific activity, 57.4 Ci/mmol) at a final concentration of 1 μM; cells were incubated at 25°C for the designated time; the uptake was terminated by washing cells twice in cold incubation medium, and cells were solubilized overnight in 0.25 mM NaOH. Cell-associated radioactivity was determined in cell lysates by scintillation counting and total protein content in lysates was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA). The results were expressed in nmoles of L-[^{3}H]-glutamate per milligram of cellular protein. To determine the specificity of glutamate uptake, cells were prepared for assay as described above and exposed to L-[^{3}H]-glutamate (1 μM) alone or with an inhibitor of glutamate transport L-*trans*-pyrrolidine-2,4-deoxycarbonate (L-TPDC) (250 μM), or substrate competitors L-aspartate (250 μM) or L-glutamate (250 μM). The uptake was terminated after 10 min at 25°C as described above; the results are shown in the inset in Fig. 3, expressed in nmol glutamate/mg protein/min. To determine the kinetic constants of glutamate uptake by astrocytes, cells were infected with HIV-1 as described above or control cells were cultured for 24 h, washed, and tested in triplicate for L-[^{3}H]-glutamate uptake in the presence of 0.2–1000 μM unlabeled L-glutamate and 1 μM L-[^{3}H]-glutamate. Glutamate uptake was determined after 10 min incubation. Data expressed as pm/mg protein/min were plotted versus unlabeled glutamate concentration. The Eadie–Hofstee transformation was used to determine V_{max} and K_m using SigmaPlot 5.0 (SPSS, Inc., Chicago, IL).

Northern blot hybridization

Total cellular RNA from human fetal astrocytes was extracted using Trizol. One microgram of RNAs per lane was resolved on a 1.0% agarose/formaldehyde gel, transferred to a nylon membrane, and hybridized with an α-^{32}P-labeled, DNA probe synthesized by PCR under standard conditions. Briefly, the PCR reaction mixture contained 10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2 μM each of dATP, dTTP, and dGTP, 033 μM of [α-^{32}P]dCTP, 2.0 mM $MgCl_2$, 1 unit of Taq polymerase (Promega), 50 ng of unlabeled PCR DNA fragment obtained with the same pair of primers serving as a PCR template, and 0.5 μM of relevant primers in a total reaction volume of 50 μl. The cycling parameters were 1 min at 94°C, 1 min at 50°C, and 1 min 30 s at 72°C, and the number of cycles was 35. The unincorporated dNTPs were removed by spin column chromatography (Roche). Primers for EAAT1 were as follows: (sense) 5′-GCGCGGATCCATGACTAAAAGCAATGGAGAAGAGCC-3′; (antisense) 5′-CGCGCTCGAGTCTACATCTTGGTTTCACTGTCGATG-3′. Primers for EAAT2 were as follows: (sense) 5′-GGGGGATCCCAGACCATGGCATCTAC-3′ and (antisense) 5′-GCTGGAATTCATATCCTTATTTCTCACG-3′. The primers for small ribosomal RNA S9 (RPS), used for standardization of total cellular RNA, were as follows: (sense) 5′-CCAGTGGCCCGGAGCTGGGTTTGTCG-3′ and (antisense) 5′-TCGTCGTCTCCAGCCCCAGCCCCACC-3′. The hybridization was performed in the ULTRAhyb (Ambion, Austin, TX) at 42°C overnight, and the hybridized blots were washed twice with 2× SSC/0.1% SDS, and then twice with 0.2× SSC/0.1% SDS at 42°C. The blots were autoradiographed by exposure to

X-ray film. The relative levels of different RNAs were quantified by densitometry, first by standardizing the OD readings for a given system to their respective RPS signals, and then expressing the standardized system value relative to standardized untreated control (C) taken as 1.0.

Protein analysis by immunoblotting

Cells were lysed in a buffer containing 1% Triton X-100, 50 mM Tris–HCl, pH 7.8, 5 mM EDTA, 50 mM NaCl, 5 mM lodoacetamide, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, and 14 μM pepstatin; cell lysates were standardized by total protein content, and 2–20 μg protein/lane were resolved by SDS–PAGE on 12% polyacrylamide Ready Gels (Bio-Rad) and transferred onto a 0.2 μm Trans-Blot nitrocellulose membrane (Bio-Rad). The membranes were incubated in 5% (w/v) skim milk in T-PBS (0.1% polyoxyethyline-sorbitan monolaurate in phosphate-buffered saline) and exposed to either monoclonal antibodies to α-tubulin (Sigma) or the previously described rabbit polyclonal antibodies to EAAT1 and EAAT2 (Bar-Peled et al., 1997). Bound antibodies were detected by using appropriate horseradish peroxidase conjugated secondary antibody (Amersham, Piscataway, NJ), enhanced chemiluminescence, and autoradiography as previously described (Canki et al., 2001). Total protein content in samples was verified by their α-tubulin content prior to final electrophoresis, and the relative levels of proteins in different lanes were quantified by densitometry as described for RNA analyses above.

Detection of TNF-α in culture supernatants

TNF-α in culture supernatants was measured using the Quantikine ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The following control inducers were purchased: interleukin-1β (R&D Systems), lipopolysaccharide (LPS), and phorbol-12-myristate-13-acetate (PMA) (Sigma).

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